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Low-fouling surface plasmon resonance biosensor for multi-step detection of foodborne bacterial pathogens in complex food samples

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ABSTRACT

Recent outbreaks of foodborne illnesses have shown that foodborne bacterial pathogens present a significant threat to public health, resulting in an increased need for technologies capable of fast and reliable screening of food commodities. The optimal method of pathogen detection in foods should: (i) be rapid, specific, and sensitive; (ii) require minimum sample preparation; and (iii) be robust and cost-effective, thus enabling use in the field. Here we report the use of a SPR biosensor based on ultra-low fouling and functionalizable poly(carboxybetaine acrylamide) (pCBAA) brushes for the rapid and sensitive detection of bacterial pathogens in crude food samples utilizing a three-step detection assay. We studied both the surface resistance to fouling and the functional capabilities of these brushes with respect to each step of the assay, namely: (I) incubation of the sensor with crude food samples, resulting in the capture of bacteria by antibodies immobilized to the pCBAA coating, (II) binding of secondary biotinylated antibody (Ab_2) to previously captured bacteria, and (III) binding of streptavidin-coated gold nanoparticles to the biotinylated Ab_2 in order to enhance the sensor response. We also investigated the effects of the brush thickness on the biorecognition capabilities of the gold-grafted functionalized pCBAA coatings. We demonstrate that pCBAA-compared to standard low-fouling OEG-based alkanethiolate self-assemabled monolayers-exhibits superior surface resistance regarding both fouling from complex food samples as well as the non-specific binding of S-AuNPs. We further demonstrate that a SPR biosensor based on a pCBAA brush with a thickness as low as 20 nm was capable of detecting *E. coli* O157:H7 and *Salmonella* sp. in complex hamburger and cucumber samples with extraordinary sensitivity and specificity. The limits of detection for the two bacteria in cucumber and hamburger extracts were determined to be 57 CFU/mL and 17 CFU/mL for *E. coli* and 7.4×10^3 CFU/mL and 11.7×10^3 CFU/mL for *Salmonella sp.*, respectively. In addition, we demonstrate the simultaneous detection of *E. coli* and *Salmonella sp.* in hamburger sample using a multichannel SPR biosensor having appropriate functional coatings.

1. Introduction

Outbreaks of foodborne illnesses have shown that foodborne bacterial pathogens present a significant threat to public health (CDC, 2014, EFSA, 2014). Recent cases include the discovery of *Staphylococcus aureus* in ice cream (Fetsch et al., 2014), *Escherichia coli* O157:H7 in spinach and ground beef (Heaton and Jones, 2008), *Salmonella* in peanut butter (Gerner-Smidt et al., 2007), and *Listeria monocytogenes* in ready-to-eat meats (Gerner-Smidt et al., 2007). The CDC estimates that every year in the US over 48 million people are infected, resulting in 128,000 hospitalizations and 3000 deaths (CDC, 2014). Infectious doses for pathogens such as *E. coli* O157:H7 and *Salmonella* are not strictly defined and depend on the host age and health (Food and Drug Administration, 2012, Paton and Paton, 1998). Some of the published data indicates that they may be as low as 1 to 100 organisms (Food and Drug Administration, 2012, Paton and Paton, 1998), while other studies report much higher infective doses (Food and Drug Administration, 2012, Kothary and Babu, 2001). The most commonly infected foods include dairy products, beef, poultry, vegetables, and drinking water. These outbreaks demonstrate an urgent need for an improvement in the screening and diagnosis of food commodities.

Culture and colony counting methods represent the most

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frequent approaches for the detection of foodborne bacterial pathogens in standardized laboratories; however, these methods are excessively time consuming and have been reported to have limited sensitivity to diverse bacterial pathogens (Gracias and McKillip, 2004, Lazcka et al., 2007). In order to improve both sensitivity and selectivity, these approaches can be combined with polymerase chain reaction (PCR)-based techniques (Lampel et al., 2000, Li and Mustapha, 2004, Rodriguez-Lazaro et al., 2005, Simpson and Lim, 2005). Although PCR methods possess relatively high sensitivity (with LODs as low as a few CFU/mL), they require expensive equipment and are likewise time-consuming (Postollec et al., 2011). Currently, the most advanced techniques to detect bacterial pathogens in food and environmental samples are represented by both immunoassay-based methods as well as DNA and fluorescent microarrays (Call et al., 2003, Cudjoe et al., 1995, Disney and Seeberger, 2004, Mansfield and Forsythe, 2001, Yu et al., 2002). These methods are capable of relatively fast detection with respect to culture-based methods, yet they also require trained personnel and expensive equipment.

Biosensors have been shown to provide a simple, cost-effective alternative to conventional methods for the detection of foodborne pathogens and furthermore, are expected to enable the field detection of foodborne pathogens, thus providing significant decreases in the overall analysis time. To date, a variety of biosensors for the detection of bacteria have been demonstrated. These include electrical biosensors based on amperometry (Abdel-Hamid et al., 1999, Li et al., 2012, Lin et al., 2008, Perez et al., 1998) or electrochemical impedance spectroscopy (Barreiros dos Santos et al., 2013, Yang et al., 2004) as well as optical biosensors employing fluorescent labels (Ko and Grant, 2006, Rohde et al., 2015, Sanvicens et al., 2011, Yang and Li, 2006, Zordan et al., 2009) or label-free optical methods (Baccar et al., 2010, Cho et al., 2015, Linman et al., 2010, Rodriguez-Emmenegger et al., 2011a, Tawil et al., 2012, Wang et al., 2011, 2012).

Surface plasmon resonance (SPR) affinity biosensors represent the foremost label-free optical biosensor technology regarding the detection of foodborne pathogens. Typical LODs for bacteria achieved by SPR biosensors are on the order of 10^4 CFU/mL or higher for direct detection, where no preconcentration or amplification steps are employed. A lower limit of detection was demonstrated by Yazgan et al. (2014) who reported the detection of *E. coli* in buffer with a LOD of 2.5 CFU/mL through the use of modified carbohydrates as bioreceptors. Another strategy for improving LODs has been demonstrated by Torun et al., who combined an SPR biosensor with immunomagnetic separation and reported the detection of *E.coli* in buffer with a LOD of 3 CFU/mL. They also applied their method to the detection of *E.coli* in moderately complex samples (lake, river, and tap water samples); limits of detection for these matrices were not reported (Torun et al., 2012). Bouguelia et al. (2013) reported the SPR detection of various bacterial pathogens with LODs as low as ~3 CFU/mL in matrixes composed of the addition of crude food samples to growth medium. In this sensor a growth chamber was situated directly on the SPR surface, where at a temperature of 37 °C they were able to monitor the growth of each pathogen in real-time; however, the detection of lower pathogen concentrations required analysis times of up to 10 h.

One of the key challenges hindering the application of SPR biosensors for the detection of bacterial pathogens in real-world complex food samples remains interfering effects stemming from the sample matrix, particularly the non-specific adsorption of non-target molecules from the sample to the sensing surface (causing a false positive signal). Correspondingly, a number of research groups have pursued the development of advanced functional coatings in order to suppress this effect. An optimum functional coating for the biosensor-based analysis of food samples is one that combines a high number of functional bioreceptors with an ability to resist fouling from complex media (Homola, 2008, Vaisocherova et al., 2015a). Among the wide variety of previously reported low-fouling coatings, zwitterionic polymeric coatings have been recently demonstrated as a promising functionalizable low-fouling surface for label-free biosensing in complex media (Brault et al., 2010, Vaisocherova et al., 2015a, 2008). Nevertheless, the biosensor capabilities of such coatings have been primarily demonstrated in complex biological fluids (blood plasma or serum) (Banerjee et al., 2011, Vaisocherova et al., 2008); the use of these coatings has never been explored with food samples. Compared to blood plasma samples, food commodities contain diverse sets of components including lipids, proteins, saccharides, water, vitamins, minerals, and even synthetic low-molecular-weight additives with variable contents. This complexity results in diverse food sample physico-chemical properties (Sikorski, 2002), thus the design of a robust, functional, low-fouling coating for reliable biosensing in crude food samples remains challenging.

In this work, we present the first use of ultra-low fouling, functionalizable poly(carboxybetaine acrylamide) (pCBAA) brushes as a unique sensing surface for the multi-step detection of bacterial pathogens in crude hamburger and cucumber samples. We studied both the functional as well as the surface resistance capabilities of the pCBAA coatings for each step of a detection assay that included: (I.) the capture of *E. coli* O157:H7 and *Salmonella* sp. in hamburger and cucumber samples, (II.) the capture of biotinylated secondary antibody, and (III.) the signal enhancement using streptavidin-coated gold nanoparticles. We compare our results to those using a standard low-fouling carboxy-functional OEG-based AT-SAMs, and show that the pCBAA brushes have similar levels of functionalization while providing over an order of magnitude improvement in fouling resistance. Finally, we demonstrate the effectiveness of these brushes via the use of a pCBAA-based SPR biosensor for the sensitive and specific detection of low levels of *E. coli* O157:H7 and *Salmonella* sp. in complex cucumber and hamburger samples.

2. Materials and methods

2.1. Reagents and biological materials

N-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-diethylaminopropyl) (EDC) were purchased from PharmaTech, Czech Republic. Ethanol (purity ≥99.9%) was purchased from Merck, USA. The buffer solutions were prepared using Millipore Q water (18.0 MΩ. cm). Phosphate buffered saline (PBS, 0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4 at 25 °C) was prepared from a stock solution purchased from Sigma-Aldrich, Czech Republic. Tween® 20 (Tween), ethanolamine (EA), and casein blocking buffer (casein) were purchased from Sigma-Aldrich, Czech Republic. 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 8.0 at 25 °C (HEPES) and 10 mM sodium acetate buffer, pH 5.0 at 25 °C (SA) were prepared from stock solutions purchased from Sigma Aldrich, Czech Republic. The bromine-terminated alkanethiol (HS-C_{11}-OC(O)-IzoButyrate-Br), HS-C_{11}-$(EG)_4$-OH, and HS-C_{11}-EG_6-OCH_2-COOH thiols were purchased from Prochimia, Poland. The carboxybetaine acrylamide monomer (CBAA) was purchased from Zwitter Technology, Seattle, USA. The CuBr, $CuBr_2$, 2,2′-dipyridyl (BiPy), were purchased from Sigma-Aldrich, Czech Republic. Tetrahydrofuran (THF, purity ≥99.9%) was purchased from Penta, Czech Republic.

Heat-killed *Escherichia coli* O157:H7 (*E. coli*), *Salmonella sp.* (*Salmonella*), primary antibodies (Ab_1), and biotinylated secondary antibodies (Ab_2) against *E. coli* and *Salmonella* (anti-*E.coli*, b-anti-*E. coli*, anti-*Salmonella*, b-anti-*Salmonella*) were purchased from KPL, Inc., USA.

2.2. Preparation of food samples

For detection experiments involving food samples we used cucumbers purchased from a local food store and hamburger from a local fast food restaurant. The cucumber was washed with water and sliced prior to homogenization. Food homogenization was performed for 2 min using a Masticator (IUL Instruments, BioTech, Czech Republic) following a standardized procedure (Andrews et al., 1978). The samples were then centrifuged for 2 min at 1200 rpm to remove any residual large pieces of foods. The supernatant above the sediment was frozen until used. To confirm that these food extracts were free of *Salmonella* and *E. coli*, a series of culture-based reference experiments were performed in the Food control laboratory of the Police of the Czech Republic following standard protocols (ČSN ISO 7251 and ČSN EN ISO 6579).

2.3. SPR sensor

In this work we used a four-channel spectroscopic SPR sensor developed at the Institute of Photonics and Electronics (Prague, Czech Republic) (Homola et al., 2002, Vaisocherova et al., 2014) combined with a dispersionless microfluidic system (Springer et al., 2010). The sensor was equipped with a temperature controller with a baseline stability of 0.01 °C.

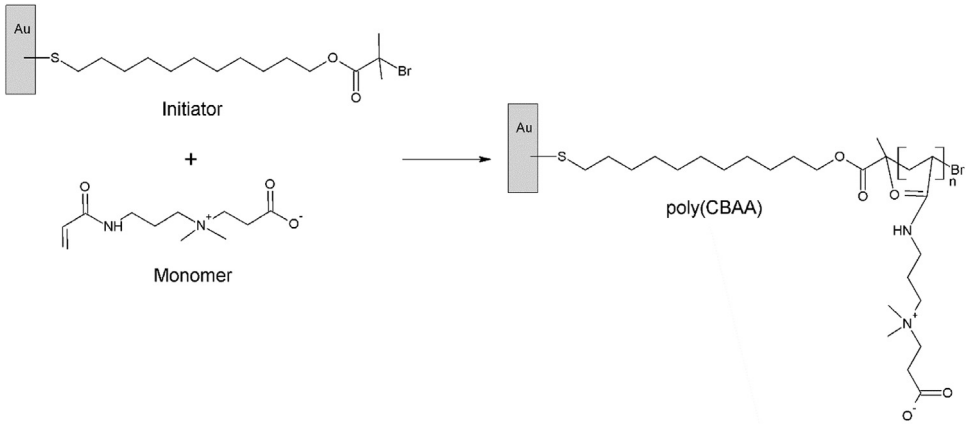
Because SPR is an evanescent field phenomenon, the sensitivity to changes at the polymer/fluid interface will decrease with increasing polymer thickness. To compensate for this effect, the SPR response was calibrated using the transfer-matrix method (Born and Emil, 1999). Briefly, we calculated the reflection spectra of a 4-layer structure consisting of BK-7 glass (RI = 1.51 RIU), a 50 nm thick gold layer, a dielectric layer corresponding to polymer brush, and water (RI = 1.33 RIU). The RI of gold and the RI and thickness of polymer layers were obtained from ellipsometry measurements, see also (Vaisocherova et al., 2008, 2014). The angle of incidence was derived from experimental values of resonant wavelengths corresponding to unmodified and activated polymer layers in contact with water. The surface sensitivity was calculated as the SPR response upon the addition of a 10 nm thick dielectric layer with RI = 1.45 RIU over the polymer brush layer. For the SPR sensor used in this study (resonant wavelength around 750 nm), a 1 nm SPR wavelength shift represents a change in the surface protein concentration of 17 ng/cm^2 (Homola, 2006, Vaisocherova et al., 2007), which assumes the same refractive index for immobilized antibodies, streptavidin, oligonucleotides, and food sample deposits. Typical correction factors for bare polymer brush thickness were 1.2 for a 20 nm thick pCBAA (RI of 1.401 RIU at 750 nm for wet surface).

2.4. Preparation and characterization of pCBAA functional coatings

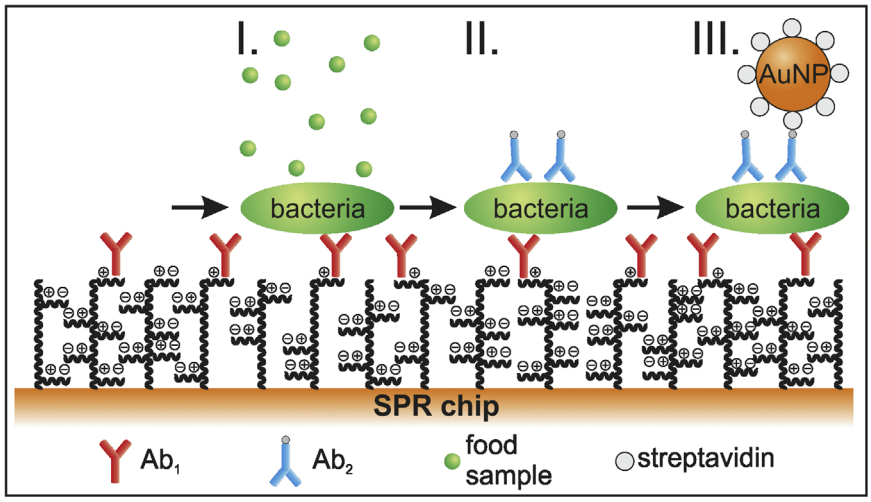
Polymer brushes of poly(3-acryloylamino-propyl)-(2-carboxyethyl)-dimethyl-ammonium) (pCBAA) were prepared on gold substrates via surface-initiated atom transfer radical polymerization (Vaisocherova et al., 2008). Using this procedure, we prepared pCBAA coatings having wet thicknesses of both ~20 and ~80 nm, whereby the pCBAA thickness was varied by changing the water/methanol ratio in the polymerization reaction (Zhang et al., 2006, 2008). The thickness of the wet brushes was measured using a previously described procedure (Vaisocherova et al., 2008, 2014) (Scheme 1).

The SPR chips coated with pCBAA brushes were stored in PBS until use, then washed with MilliQ water, dried with a stream of nitrogen, and mounted into the SPR sensor. The pCBAA surface functionalization included three steps: the activation of carboxyl groups by NHS/EDC chemistry, the covalent attachment of antibodies to activated groups, and the deactivation of residual active groups (Vaisocherova et al., 2014, 2015b). Surface carboxylates were converted to active esters by injection of a freshly prepared solution of *N*-hydroxysuccinimide (NHS, 0.1 M) and *N*-ethyl-*N*′-(3-diethylaminopropyl) (EDC, 0.5 M) in MilliQ water for 15 min at a flow rate of 5 μl/min. Antibodies (25 μg/mL) diluted in HEPES (10 mM, pH 8.0) were filtered using a syringe filter (0.22 μm, TPP Switzerland) and immobilized on the activated surface in a flow-through format at a flow rate of 20 μL/min for 20 min. Any residual active esters were then deactivated using consecutive injections of PBS and SA. The amount of immobilized antibodies on each surface was determined from the difference between the final and initial sensor response.

This surface functionalization protocol was adjusted in order to achieve a maximum loading capacity of immobilized Ab_1 while maintaining a high resistance to fouling. For purposes of comparison we measured the fouling resistance of both the pCBAA brushes as well as surfaces composed of standard low-fouling carboxy-functional mixed oligo(ethylene glycol) alkanethiolate self-assembled monolayers (AT-SAMs); the preparation and functionalization of the AT-SAMs used herein is described in Supplementary Material. Fouling levels were measured by flowing food samples (45 μL/min, 25 °C, 15 min) over both Ab_1-functionalized pCBAA (~20 nm wet thickness) and AT-SAM surfaces having Ab_1 surface coverages in the range of ~175–275 ng/cm^2. The fouling characteristics were determined after washing the surface with PBS buffer for 10 min.



Scheme 1. Synthesis of pCBAA brushes via surface initiated and surface-initiated atom transfer radical polymerization.



Scheme 2. Scheme of the three-step assay for the detection of bacterial pathogens in food samples.

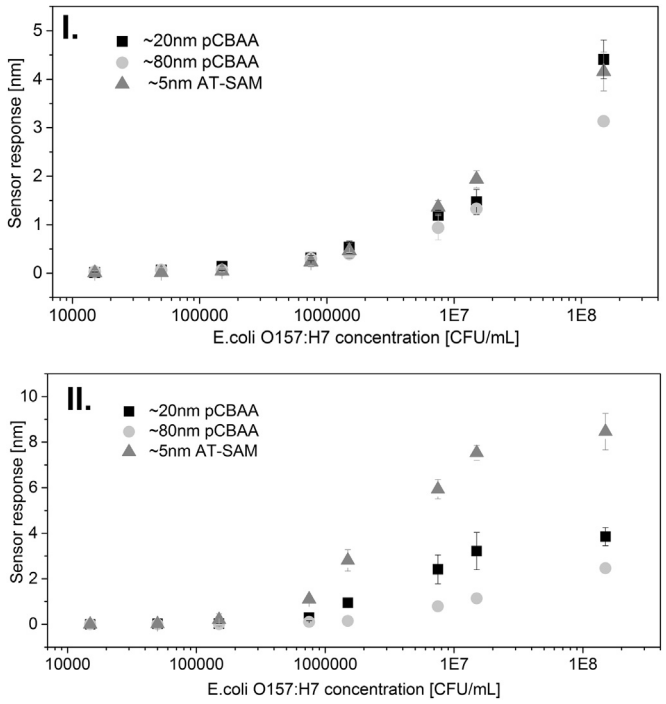


Fig. 1. Sensor response to the capture of the *E.coli* O157:H7 (I.) and Ab_2 (II.) regarding 20 and 80 nm thick pCBAA brushes and a 5 nm thick AT-SAM layer. Response of sensors employing alkanethiolate self-assembled monolayers (AT-SAM) are shown for comparison.

2.5. SPR biosensor-based detection of bacteria

The detection of bacteria spiked in hamburger and cucumber samples was performed for *E.coli* and *Salmonella* concentrations ranging from 1.5×10^1 to 1.5×10^7 CFU/mL and 2.5×10^2 to 2.5×10^7 CFU/mL, respectively. Pathogens spiked in PBS buffer were only used in experiments to characterize the effect of the pCBAA brush thickness on the biosensor biorecognition capabilities. The format of the detection experiments is shown in Scheme 2. All experiments were conducted at 25 °C and proceeded as follows: (I.) food samples spiked with bacteria were flowed along surfaces previously functionalized with respective Ab_1 (45 μL/min, 15 min), (II.) followed by an injection of Ab_2 (5 μg/mL, 20 μL/min,15 min), and finally (III.) followed by an injection of streptavidin-coated spherical gold nanoparticles (S-AuNPs) having an OD of 1 (20 μL/min,25 min) in PBS buffer with casein (0.01% w/v) and Tween (0.01% w/v) (Springer and Homola, 2012). Each data point was obtained using data from at least three individual pCBAA coatings and up to eight sensing channels. The procedure for the synthesis and functionalization of S-AuNPs with a diameter of ~35 nm was taken from (Springer et al., 2014), and is also described in the Supplementary Material.

Food samples without bacteria (spiked with the same amount of bacteria-free buffer) were injected in a single channel for each coating to detect any potential non-specific binding of S-AuNPs (blank sample). The signal from the reference channel was subtracted from those provided by the detection channels. The limit of detection (LOD) was calculated using a "blank+3 × SD" approach.

The simultaneous detection of *E.coli* and *Salmonella* in hamburger samples was performed under the same conditions as used for the detection of individual pathogens. Each sensor chip contained two detection channels and two reference channels, where each pair was functionalized with either anti-*E.coli* or anti-*Salmonella*. Three different mixtures of *E.coli* and *Salmonella* in hamburger samples were analyzed. The concentrations of *E.coli* and *Salmonella* spiked in these samples were 7.5×10^2 and 2.5×10^7 CFU/mL (MIX-1), 2.5×10^6 and 1.3×10^6 CFU/mL (MIX-2), and 7.5×10^9 and 1.3×10^4 CFU/mL (MIX-3), respectively. The results obtained from the measurements on bacteria mixtures were compared with those obtained for individual pathogens.

3. Results and discussion

3.1. Performance of pCBAA coatings for the multi-step detection of bacterial pathogens in complex food matrices

We measured the effect of the thickness of gold-grafted zwitterionic pCBAA coatings on the biorecognition capabilities of the antibody-functionalized surface. Specifically, we measured the response of the anti-*E.coli*-functionalized pCBAA-coated sensor to both *E.coli* in buffer as well as to the respective secondary antibody (Ab_2) for two different coating thicknesses (between 20 nm and 80 nm). Due to the rather limited control over the thickness of pCBAA grafted from gold substrates by SI-ATRP (Rodriguez-Emmenegger et al., 2011b, 2011c), we selected these two thicknesses as distinct enough to be reproducibly prepared.

These results are shown in Fig. 1; for purposes of comparison we have included data regarding the use of a thin AT-SAM (~5 nm) (Prime and Whitesides, 1991, Ulman, 1996). It can be seen (upper

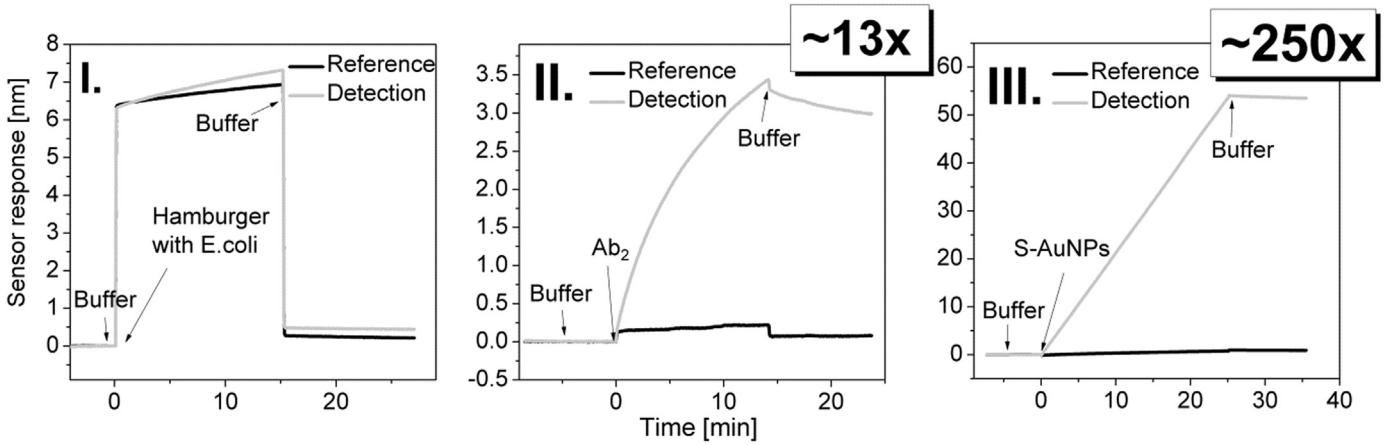


Fig. 2. Typical SPR sensorgrams obtained for the detection of *E. coli* O157:H7 (7.5×10^5 CFU/mL) in hamburger sample for each step of the detection assay as depicted in Scheme 2. The sensor response enhancement is given in the boxes.

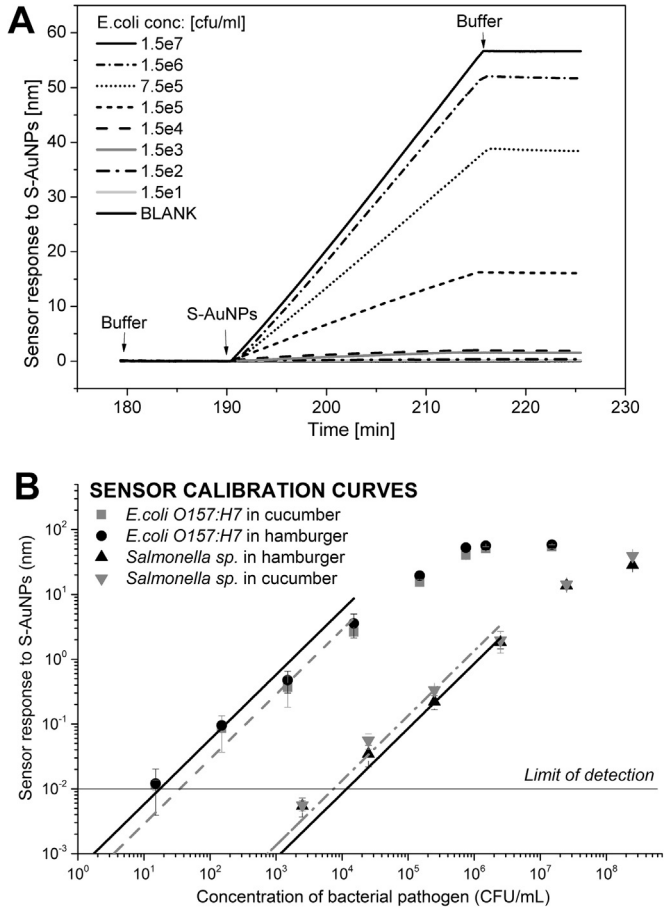


Fig. 3. (A) SPR sensor response to the binding of S-AuNPs in step (III) of the assay for the detection of *E.coli O157:H7* in cucumber sample. (B) Calibration curves for *E. coli* and *Salmonella sp.* in both hamburger and cucumber samples.

graph) that the capture of *E.coli* (Scheme 2/I.) was not significantly influenced by the pCBAA thickness, whereas the effect of the pCBAA thickness was more pronounced in the sensor response to the secondary antibody (Scheme 2/II.). This effect may be attributed to the differences in stearic hindrance between the binding of secondary antibody to bacterium bound to brushes of different thicknesses; however, this effect cannot be properly accounted for using the calibration methods used in this study (Homola, 2008). Nevertheless, the discrepancy between the sensor response of all three layers decreases as the concentration of *E.coli* decreases.

In contrast to the pCBAA polymer brushes, the AT-SAMs coatings provided a very limited resistance to fouling from food samples. Specifically, the respective fouling levels from cucumber and hamburger samples for the AT-SAMs were 22.3 ± 5.2 ng/cm^2 and 69.7 ± 11.9 ng/cm^2, whereas the same levels for ~20 nm pCBAA brushes were 6.2 ± 1.7 ng/cm^2 and 3.4 ± 1.3 ng/cm^2. The superior surface resistance to fouling for pCBAA compared to AT-SAMs is in agreement with previous studies on the fouling from bodily fluids (Vaisocherova et al., 2015a, 2014, 2015b). The average Ab_1 immobilization levels achieved for pCBAA (~20 nm) were 221 ± 36 ng/cm^2 and 209 ± 51 ng/cm^2 for anti-*E.coli* and anti-*Salmonella*, respectively; the same values for AT-SAMs were 200 ± 39 ng/cm^2 and 169 ± 13 ng/cm^2. Fouling levels from hamburger and cucumber samples on anti-*E.coli*-functionalized pCBAA surface were thus below 2.8% of the anti-*E.coli* immobilization level. This ratio is significantly lower than those observed on AT-SAMs, which had fouling ratios of 11% and 35% for cucumber and hamburger, respectively. Based on these results, a pCBAA coating with a thickness of ~20 nm was used for all further bacteria detection experiments. This lower thickness is also beneficial from the perspective of the SPR surface sensitivity (Homola, 2008).

We further compared the nonspecific binding of S-AuNPs to anti-*E.coli*-functionalized pCBAA (having a thickness of ~20 nm) with an AT-SAM surface (see Figure S-2 in Supplementary Material). We found that the pCBAA coatings had nearly an order of magnitude better performance. Specifically, based on SPR measurements with *E. coli* O157:H7 spiked in hamburger we found that the levels of nonspecific binding of S-AuNPs to pCBAA and AT-SAM coatings (functionalized with anti-*E.coli* O157:H7 followed with *E. coli* O157:H7 detection in hamburger) were between 0.2 nm and 1.6 nm, respectively. For the blank surfaces (which involve all three detection steps, where bacteria-free food samples were injected instead of bacteria-spiked food sample), the nonspecific binding of S-AuNPs on pCBAA and AT-SAM coatings were 0.9 and 89 nm, respectively. This clearly implies that the nonspecific binding of secondary biotinylated antibody (b-anti-*E.coli* O157:H7) to AT-SAM significantly contributed to a very high level of S-AuNPs in a reference blank surface. Hence, the combination of the high surface resistance to binding of both Ab_2 and S-AuNPs makes the pCBAA coating superior over the AT-SAM.

3.2. Detection of individual foodborne pathogens in food samples

We further characterized the high functional and surface-resistance capabilities of pCBAA in a multi-step detection assay through the use of a SPR biosensor based on pCBAA with a thickness

Table 1
SPR response to S-AuNPs obtained from the simultaneous detection of *E.coli* and *Salmonella* in hamburger samples. The results were compared to results determined from the respective pathogen's calibration curve ("single pathogen response").

Mix	Bacterial pathogen	Spiked concentration (CFU/mL)	Sensor response (nm)	Single pathogen response (nm)	Recovery (%)
1.	*E.coli* O157:H7	7.5×10^2	0.026 ± 0.01	0.025 ± 0.008	104
	Salmonella sp.	2.5×10^7	10.5 ± 1.0	10.5 ± 1.1	100
2.	*E.coli* O157:H7	7.5×10^3	0.09 ± 0.03	0.10 ± 0.02	90
	Salmonella sp.	1.3×10^6	0.7 ± 0.1	0.8 ± 0.2	95
3.	*E.coli* O157:H7	7.5×10^6	95 ± 20	111 ± 15	86
	Salmonella sp.	1.3×10^4	0.010 ± 0.005	0.12 ± 0.005	83

of 20 nm. Using this three-step assay, two model foodborne pathogens (*E. coli* O157:H7 and *Salmonell*a sp.) were detected in crude cucumber and hamburger samples (Scheme 2). Typical sensor data obtained during each step of the assay for both the measuring and reference channel are shown in Fig. 2. Fig. 3A shows the SPR sensor response to the binding of S-AuNPs during step (III) for various concentrations of *E.coli* in cucumber samples. The calibration curves for *E.coli* and *Salmonella sp.* in both hamburger and cucumber samples are shown in Fig. 3B. The limits of detection were determined to be 17 CFU/mL and 57 CFU/mL for *E.coli* in hamburger and cucumber samples, respectively, and 11.7×10^3 CFU/mL and 7.4×10^3 CFU/mL for *Salmonella* in hamburger and cucumber samples, respectively. The discrepancies between the LODs obtained for *E. coli* and *Salmonella* were most likely due to the differences in the affinity and specificity of the respective antibodies.

Including sample preparation, the detection times for the results herein were ultimately shorter than 80 min, which is remarkably shorter than the time required by standard culture-based techniques (Gracias and McKillip, 2004, Lazcka et al., 2007). The LODs demonstrated herein for *E. coli* were either comparable, or up to one order of magnitude higher, than those of previous reports that are regarded as the most sensitive SPR biosensors for bacterial pathogen detection (Bouguelia et al., 2013, Torun et al., 2012, Yazgan et al., 2014); however, those LOD values regard the detection of pathogens either in buffer or solutions of medium complexity such as tap water. In addition, the LODs for the majority of previous biosensor-based studies reporting the detection of pathogens in complex food samples – including those reporting very low LODs – were not determined through the measurement of sensor calibration curves in said complex media (see also Table S-1 in Supplementary Material). To the best of our knowledge this study represents the first time-efficient (< 80 min) SPR-based biosensor detection of bacterial pathogens in crude food samples with a LOD as low as tens of bacteria per 1 mL of complex food extract without using bacteria enrichment. Furthermore, it is envisioned that these limits of detection can be further improved by adjusting the respective detection assay conditions (e.g. Ab_2 concentrations) or by employing synthetic bioreceptor alternatives to the primary or secondary antibodies (Yazgan et al. 2014).

3.3. Simultaneous detection of E.coli and salmonella in crude food samples

This biosensor was also utilized for the simultaneous detection of *E.coli* and *Salmonella* in hamburger samples. Specifically, crude hamburger samples with three different combinations of *E.coli* and *Salmonella* concentrations were analyzed. A series of control SPR experiments confirmed that there is no significant cross-reactivity between the antibodies (between Ab_1 and Ab_2) and bacterium used in this study (data not shown). The results of the simultaneous detection experiments are shown in Table 1. We compared the sensor response for each pathogen in mixed samples with the single pathogen responses given by the respective calibration curves. It can be seen that there was a good agreement between the results obtained in the simultaneous detection experiments and those obtained for the detection of individual pathogens. This indicates that the sensor provides sufficient specificity and indeed allows for the simultaneous detection of the two bacterial pathogens.

4. Conclusions

This study demonstrates the use of an ultra-low fouling functionalizable pCBAA coating as a unique biosensor surface platform providing both high functional and surface-resistance capabilities towards the multi-step detection of *E. coli* O157:H7 and *Salmonella* sp. in crude hamburger and cucumber samples. Specifically, the antibody-functionalized pCBAA coatings with a thickness as low as ~20 nm exhibited a high resistance to (i) fouling from hamburger and cucumber samples, to (ii) the nonspecific binding of secondary biotinylated antibodies, and to (iii) the nonspecific binding of streptavidin-functionalized gold nanoparticles. The fouling levels for the pCBAA brushes were up to two orders of magnitude lower when compared to standard low-fouling carboxy-functional oligo (ethylene glycol)-based AT-SAMs. Finally, a SPR biosensor based on a pCBAA coating was capable of the time-efficient (< 80 min) detection of *E. coli* and *Salmonella* with extraordinary sensitivity and specificity: the limits of detection for *E. coli* were 57 CFU/mL and 17 CFU/mL for cucumber and hamburger samples, respectively. In future we plan to further optimize the functional coatings and assay to achieve even higher sensitivity and reproducibility to enable rapid parallelized detection of multiple pathogens in food samples.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.01.040.

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